Biochemical and Biophysical Research Communications 460 (2015) 1041–1046

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Reovirus double-stranded RNA genomes and polyI:C induce down-regulation of hypoxia-inducible factor 1α

Takuma Hotani [a], Masashi Tachibana [a], Hiroyuki Mizuguchi [a, b, c, d], Fuminori Sakurai [a, e, *]

[a] Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan
[b] Laboratory of Hepatic Differentiation Research, National Institute of Biomedical Innovation, Osaka, Japan
[c] Center for Advanced Medical Engineering and Informatics, Osaka University, Osaka, Japan
[d] Laboratory of iPS Research, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan
[e] Laboratory of Regulatory Sciences for Oligonucleotide Therapeutics, Clinical Drug Development Unit, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan

ARTICLE INFO

Article history:
Received 6 March 2015
Available online 2 April 2015

Keywords:
Reovirus
HIF-1α
dsRNA
Cathepsin
polyI:C

ABSTRACT

Reovirus has genomes consisting of 10-segmented double-stranded RNAs, and have received much attention as an oncolytic virus. A previous study reported that reovirus down-regulates hypoxia-inducible factor 1α (HIF-1α) protein levels following infection in tumor cells, which contributes to the antitumor effects of reovirus; however, the mechanism remains to be elucidated. In this study, we examined which virus component was involved in reovirus-mediated down-regulation of HIF-1α. Reovirus induced significant down-regulation of HIF-1α protein levels in not only reovirus-permissive tumor cells but also reovirus–resistant tumor cells. UV-inactivated reovirus also induced a reduction in HIF-1α protein levels. These data indicate that reovirus induces HIF-1α down-regulation independently of virus replication. Furthermore, transfection with not only reovirus genomes but also polyI:C efficiently induced HIF-1α down-regulation in a manner similar to reovirus, indicating that double-stranded reovirus RNA genomes are a key component for HIF-1α down-regulation. Reovirus-mediated HIF-1α down-regulation was inhibited when tumor cells were pretreated with inhibitors of cathepsins B and L, which play a crucial role in endo-lysosomal escape of virions to the cytoplasm. These data suggest that endo-lysosomal escape of reovirus genome into the cytoplasm is crucial for HIF-1α down-regulation; however, the retinoic acid-inducible gene-I (RIG-I) or interferon-β promoter stimulator-1 (IPS-1), which are involved in reovirus genome-induced innate immunity in the cytoplasm, did not play a crucial role in reovirus-mediated HIF-1α reduction.

1. Introduction

Mammalian orthoreovirus (reovirus), which possesses a non-enveloped icosahedral capsid enclosing a 10-segmented double-stranded RNA (dsRNA) genome, is ubiquitous but generally without clinical symptoms, except in the very young. There are three reovirus serotypes, with the prototype strains being type 1 Lang (T1L), type 2 Jones (T2J), and the two serotype 3 prototypes: type 3 Abeney (T3A) and type 3 Dearing (T3D) [1]. T3D (hereafter reovirus) has been actively studied as an oncolytic virus, which specifically replicates in tumor cells but not in normal cells. Clinical trials of cancer therapies using reovirus, including phase III clinical trials, are ongoing internationally [2,3].

Reovirus infects cells *via* several steps. First, the reovirus particles bind to cellular receptors of reovirus, including junction adhesion molecule-A (JAM-A) and Nogo receptor NgR1 [4,5], and then they are internalized into endosomes. The outer capsid proteins are degraded in the lysosomes by lysosomal proteases, cathepsins B and L, leading to the escape of virions into the cytoplasm [6]. In tumor cells, the proteolytic activities of cathepsins B and L are often elevated, compared with normal cells, leading to efficient invasion into the cytoplasm in tumor cells [7,8]. In addition, efficient translation of reovirus genomes occurs in Ras-activated tumor cells due to the inactivation of dsRNA-dependent protein kinase

* Corresponding author. Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita-City, Osaka 565-0871, Japan. Fax: +81 6 6879 8186.
E-mail address: sakurai@phs.osaka-u.ac.jp (F. Sakurai).

http://dx.doi.org/10.1016/j.bbrc.2015.03.147
0006-291X/

(PKR), leading to efficient production of the progeny virus in tumor cells [9]. Reovirus also exhibits antitumor effects *via* activation of antitumor immune responses. Reovirus genomes are recognized by retinoic acid-inducible gene-1 (RIG-I) and melanoma differentiation associated gene 5 (MDA5) in the cytoplasm, leading to induction of innate immunity [10]. Reovirus has been shown to induce activation of immune cells, including $CD8^+$ cells, NK cells, and dendritic cells, in mice [11].

In addition to the mechanisms of the reovirus-mediated antitumor effects described above, reovirus has been demonstrated to induce down-regulation of hypoxia inducible factor-1α (HIF-1α), which is a transcriptional factor driving the expression of a variety of genes crucial for cell survival and growth under hypoxia, in several types of tumor cell lines in a proteasome-dependent manner [12,13]. HIF-1α is highly expressed in tumors because hypoxia is induced within tumors due to rapid tumor cell growth and immature vascular formation. HIF-1α promotes tumor progression by elevating the expressions of various genes, including vascular endothelial growth factor (VEGF), multidrug resistance 1 (MDR1), and glucose transporter 1 (GLUT1) [14–16]. HIF-1α is a promising target for cancer therapy. Small interfering RNA (siRNA)-mediated knockdown of HIF-1α has been shown to significantly suppress tumor growth [17]. Reovirus-induced down-regulation of HIF-1α in tumor cells contributes to the antitumor activity of reovirus. However, the mechanism remains to be clarified.

In this study, we examined the mechanism of reovirus-mediated down-regulation of HIF-1α. Our results showed that reovirus induced HIF-1α down-regulation independently of virus replication. Among the virus components, double-stranded reovirus RNA genomes play a crucial role in HIF-1α down-regulation. These findings should provide important clues toward elucidation of the mechanism of reovirus-mediated HIF-1α down-regulation and cancer therapies using reovirus.

2. Materials and methods

2.1. Cell lines and virus

Human and mouse cultured cells were maintained in an appropriate culture medium containing 5% or 10% fetal bovine serum (FBS) and antibiotics at 37 °C in a 5% CO_2 atmosphere. For hypoxic exposure, cells were incubated in a hypoxic chamber (Veritas Co., Tokyo, Japan) in the presence of 1% O_2, 5% CO_2/balance N_2 at 37 °C. Reovirus (kindly provided by Dr. T. Etoh and A. Nishizono, Oita University, Oita, Japan) was grown in L929 cells and purified by CsCl ultracentrifugation, followed by overnight dialysis. Biological titers of reovirus were determined by a plaque-forming assay using L929 cells. For inactivation treatment, reovirus was irradiated with UV for 1 h. Inactivation of reovirus was confirmed by cytotoxic effects on L929 cells.

2.2. Treatment with reovirus, reovirus genome, and polyI:C

Human tumor cell lines were seeded on a 6-well plate at 1×10^5 cells/well or a 24-well plate at 4×10^4 cells/well. On the following day, reovirus was added to the cells at the indicated multiplicities of infection (MOIs). The cells were incubated under hypoxic conditions for 24 or 48 h after the addition of reovirus, then recovered and subjected to western blotting analysis. For transfection with reovirus genomes and polyI:C (Sigma, St. Louis, MO), the cells were seeded as described above and transfected with reovirus genomes and polyI:C using Lipofectamine RNAiMAX (Life Technologies, Carlsbad, CA). Reovirus genomes were recovered as described below. Cells were then cultured under hypoxic conditions and recovered 24 h after transfection for western blotting analysis.

2.3. Western blotting analysis

Cell lysates were prepared 24 or 48 h after the addition of reovirus by adding RIPA buffer (Thermo Scientific, Rockford, IL) containing Protease Inhibitor Cocktail (Sigma), DL-Dithiothreitol solution (Sigma), and PMSF (Sigma) to the cells. The lysates were analyzed by SDS-PAGE. The proteins were electro-transferred onto a PVDF membrane. Western blot assays were carried out by using anti-HIF-1α monocloal antibody (clone: 610959, 1:1000; BD Biosciences, San Jose, CA), anti-reovirus σ3 monocloal antibody (clone: 4F2, 1:2000; Developmental Studies Hybridoma Bank, Iowa City, IA) and anti-β-Actin monocloal antibody (clone: AC-15, 1:10,000; Sigma) as a primary antibody and Chemi-Lumi One Super (Nacalai Tesque, Kyoto, Japan). Images were captured using an LAS-4000 system (Fujifilm, Tokyo, Japan).

2.4. Gel extraction of the reovirus genome

Reovirus genome RNA was extracted from purified reovirus particles using an RNeasy Mini Kit (QIAGEN, Venlo, NL) according to the manufacturer's instructions. Each segment of the reovirus genome RNA was separately extracted after gel electrophoresis using a QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer's instructions.

2.5. Pretreatment with cathepsin inhibitors and small interfering RNAs (siRNAs)

Cells were pretreated with a cathepsin B inhibitor, CA-074 (Sigma), at 50 μM and cathepsin L inhibitor III (Millipore, Darmstadt, Germany) at 10 μM. Following a 30-min incubation, the cells were infected with reovirus as described above. For knockdown of RIG-I and interferon-β promoter stimulator-1 (IPS-1), cells were transfected with siRNAs targeting human RIG-I (Dharmacon, Waltham, MA) and human IPS-1 (Gene Design Inc., Osaka, Japan), and with negative control siRNA (QIAGEN) using Lipofectamine RNAiMAX (Invitrogen). The target sequence of an siRNA against IPS-1 was as follows: 5′-TCG TCC GCG AGA TCA ACT A-3'. Following a 48-h incubation, cells were transfected with reovirus genomes and cultured under hypoxic conditions as described above. HIF-1α protein levels were analyzed by western blotting 24 h after reovirus genome transfection as described above.

3. Results

3.1. Reovirus down-regulates HIF-1α protein levels in both reovirus-permissive and -resistant tumor cells

In order to examine the correlation between the reovirus-mediated down-regulation of HIF-1α and the permissiveness of tumor cells to reovirus-mediated cell killing, HIF-1α protein levels in several tumor cell lines were evaluated following treatment with reovirus. We previously demonstrated that HepG2 and H1299 cells exhibited efficient replication of the reovirus genome and the low cell viability following addition of reovirus, while A549 and MDA-MB-231 cells were refractory to reovirus [18]. HIF-1α expression levels were decreased in not only reovirus-permissive cells (HepG2 and H1299 cells) but also −resistant cells (A549 and MDA-MB-231 cells) following treatment with reovirus, especially at an MOI of 20 (Fig. 1A). These results indicate that reovirus induces down-regulation of HIF-1α in both reovirus-permissive and -resistant tumor cells.

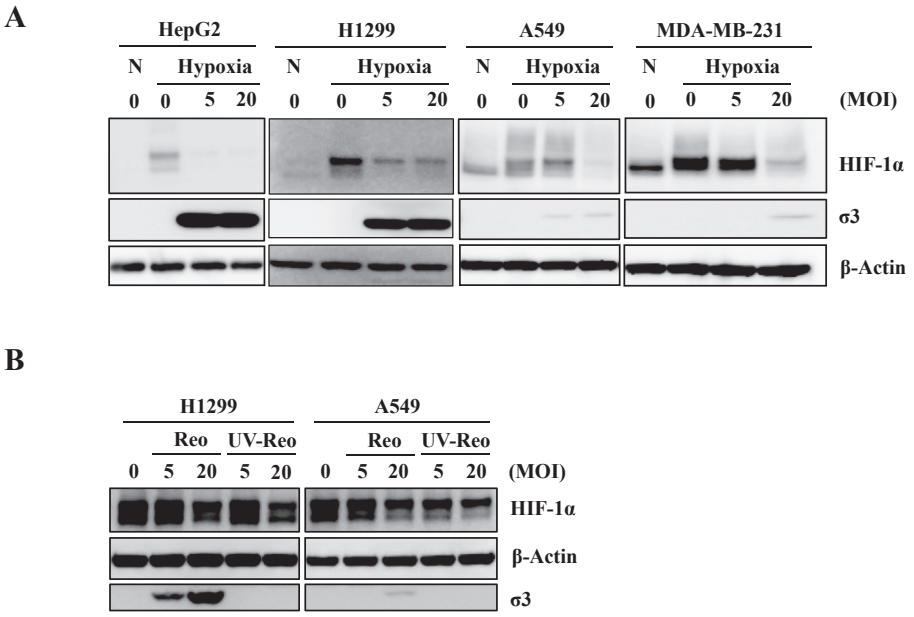

Fig. 1. HIF-1α protein levels in human cultured cells following the addition of live reovirus or UV-inactivated reovirus under hypoxic conditions. (A) HepG2, H1299, A549, and MDA-MB-231 cells were treated with reovirus at the indicated MOIs for 48 h. (B) H1299 and A549 cells were treated with live reovirus (Reo) or UV-inactivated reovirus (UV-Reo) at the indicated MOIs. Cells were cultured under normoxic and hypoxic conditions for 24 h after addition of reovirus, followed by western blotting. N; normoxic conditions.

3.2. Virus replication is dispensable for reovirus-mediated HIF-1α down-regulation

Next, in order to examine whether reovirus replication was required for reovirus-mediated HIF-1α down-regulation, H1299 and A549 cells were treated with UV-inactivated reovirus. UV-induced inactivation of reovirus was confirmed in a cell killing assay using L929 cells, which reovirus efficiently infects and lyses (Supplemental Fig. 1). UV-inactivated reovirus mediated down-regulation of HIF-1α at a level comparable to, or slightly higher than, that mediated by live reovirus in H1299 and A549 cells (Fig. 1B). These results indicate that reovirus replication is not required for the down-regulation of HIF-1α protein levels.

3.3. Reovirus genomes are a key factor for reovirus-mediated down-regulation of HIF-1α

In order to investigate which virus components were involved in reovirus-mediated down-regulation of HIF-1α expression, H1299 and A549 cells were transfected with reovirus genomes. HIF-1α protein levels in H1299 and A549 cells following reovirus genome transfection were dose-dependently reduced (Fig. 2A). In addition, a synthetic dsRNA, polyI:C, also induced down-regulation of HIF-1α at a level comparable to reovirus genomes. These results indicate that double-stranded reovirus RNA genomes are a key component for reovirus-mediated HIF-1a down-regulation. Cell viabilities were not significantly reduced following transfection (data not shown).

Next, in order to examine which segments of reovirus genomes were crucial for reovirus-mediated HIF-1α down-regulation, each segment was recovered and transfected in H1299 and A549 cells. Reovirus genomes consist of 10 segments that are classified into three size classes: large (3.9 kb), medium (2.2–2.3 kb), and small segments (1.2–1.4 kb). HIF-1α protein levels were significantly reduced following transfection with each genome segment (Fig. 2B). No differences were detected in the levels of reduction of HIF-1α between each segment and the total reovirus genomes. These results indicate that all the segments of reovirus genomes contribute to the reovirus-mediated down-regulation of HIF-1α.

3.4. Cathepsins B and L are important for reovirus-mediated down-regulation of HIF-1α

In order to examine whether cathepsins B and L are crucial for reovirus-mediated down-regulation of HIF-1α expression, H1299 cells were pre-treated with CA-074, which is a cathepsin B inhibitor, and cathepsin L inhibitor III and subsequently treated with reovirus. Inhibitors of cathepsins B and L block the penetration of virus particles from the lysosomes into cytosol because cathepsins B and L are proteases crucial for the degradation of outer capsid proteins of reovirus and efficient penetration of virions into the cytoplasm [6]. The inhibition of reovirus infection by these cathepsin inhibitors was demonstrated by a reduction in the reovirus protein σ3 levels (Fig. 3). Reovirus-mediated down-regulation of HIF-1α did not occur when the cells were pre-treated with cathepsin L inhibitor III. Although cathepsin B inhibitor CA-074, when administered singly, induced down-regulation of HIF-1α, further down-regulation of HIF-1α was not induced following treatment with reovirus in the cells pre-treated with CA-074. These results indicate that degradation of the outer capsid proteins by cathepsins B and L and subsequent penetration into the cytosol are crucial for the reovirus-induced down-regulation of HIF-1α protein levels.

3.5. The RIG-I/IPS-1 pathway is not involved in reovirus-induced down-regulation of HIF-1α

Next, in order to examine whether reovirus-induced innate immune responses were implicated in the down-regulation of HIF-1α protein levels, RIG-I and IPS-1, which are a pattern recognition receptor recognizing reovirus genomes and the down-stream signal molecule, respectively, were knocked down by siRNA transfection, followed by transfection with reovirus genomes. RIG-I recognizes reovirus genomes in the cytoplasm and triggers type I IFN induction *via* IPS-1 [19,20]. The RIG-I- and IPS-1-targeted siRNAs used in this study mediated more than 70% knockdown in the mRNA levels of both genes (Supplemental Fig. 2) [20], as we previously reported [20]. Significant reductions in HIF-1α protein

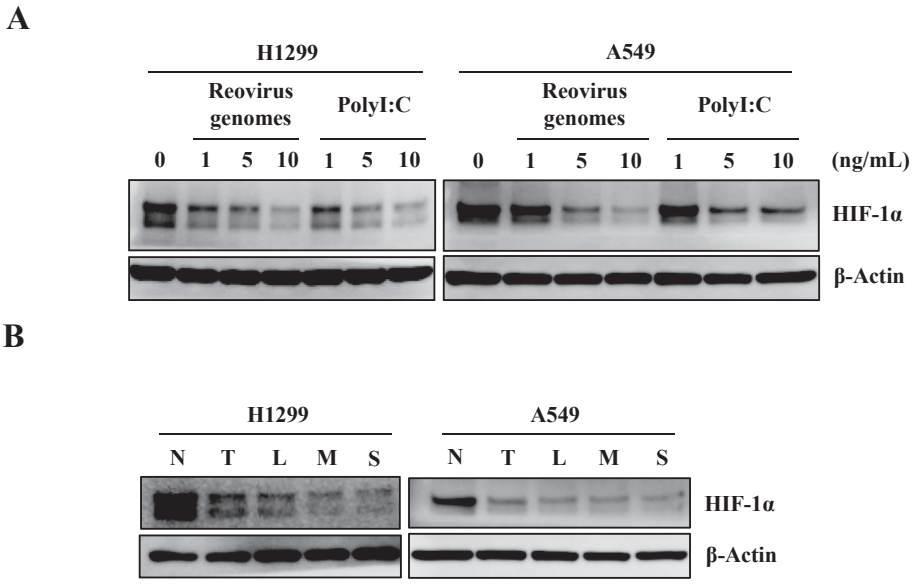

Fig. 2. HIF-1α protein levels in human cultured tumor cells following reovirus genome transfection. (A) H1299 and A549 cells were transfected with reovirus genomes or polyI:C at the indicated doses. (B) H1299 and A549 cells were transfected with each segment of reovirus genomes (N: non-treatment; T: total genomes; L: large segments; M: medium segments; S: small segments) at 4 ng/mL. Cells were cultured for 24 h under hypoxic conditions, followed by western blotting analysis.

levels were observed following transfection with reovirus genomes in the cells pre-treated with siRNAs targeting RIG-I and IPS-1 (Fig. 4). These results indicate that reovirus genome-mediated innate immune responses *via* RIG-I or IPS-I are not involved in the down-regulation of HIF-1α protein levels following treatment with reovirus.

4. Discussion

HIF-1α plays a crucial role in the growth and survival of tumors. Down-regulation of HIF-1α leads to inhibition of tumor progression because HIF-1α promotes angiogenesis, metabolic adaption, invasion, and metastasis *via* binding to hypoxia response elements in the promoter region of target genes [21]. Previous studies have demonstrated that reovirus infection resulted in a down-regulation of HIF-1α in human cancer cell lines [12,13]. However, the mechanism underlying this effect has not been elucidated. In this study, we found that double-stranded reovirus RNA genomes induced down-regulation of HIF-1α.

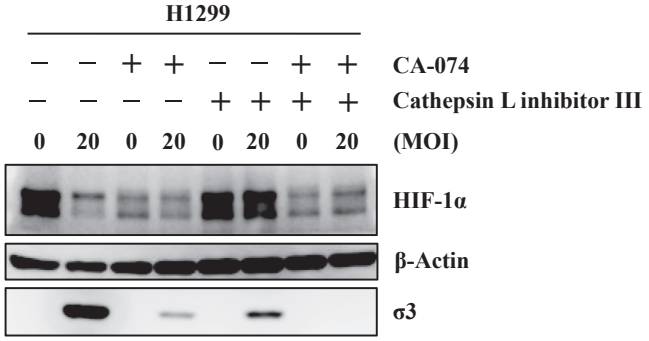

Fig. 3. HIF-1α protein levels in human cultured tumor cells pre-treated with inhibitors of cathepsins B and L following treatment with reovirus. H1299 cells were pre-treated with CA-074 (50 μM) and cathepsin L inhibitor III (10 μM) for 30 min. Cells were treated with reovirus at an MOI of 20, followed by incubation under hypoxic conditions for 24 h. HIF-1α protein levels were evaluated by western blotting analysis.

Previous studies reported the reovirus-mediated down-regulation of HIF-1α in tumor cell lines [12,13]; however, it remained to be clarified whether HIF-1α was down-regulated by reovirus even in tumor cells refractory to reovirus-induced cell killing (Fig. 1A). This study clearly demonstrated that reovirus induced HIF-1α down-regulation at an almost comparable level in both cells susceptible and refractory to reovirus-induced cell killing, despite the fact that the replication of virus genomes in reovirus-permissive tumor cells was more efficient than that in reovirus-resistant tumor cells [18]. Down-regulation of HIF-1α expression was observed in tumor cells treated with not only live reovirus but also UV-inactivated reovirus (Fig. 1B). These results indicate that the replication of reovirus genomes is dispensable for HIF-1a down-regulation. UV radiation produced shortened and fragmented virus genomes, and even fragmented virus genomes efficiently induced HIF-1α down-regulation.

Cathepsins B and L are crucial for proteolytic disassembly of the outer capsid proteins of reovirus, which allows endo-lysosomal escape into the cytoplasm. Inhibitors of cathepsins B and L significantly inhibited not only reovirus-mediated tumor cell killing but also reovirus-mediated type I interferon production *via* the RIG-I/IPS-1 pathway due to the inhibition of endo-lysosomal escape of the reovirus genome into the cytoplasm [18,20]. Reovirus-mediated down-regulation of HIF-1α protein levels was not observed when

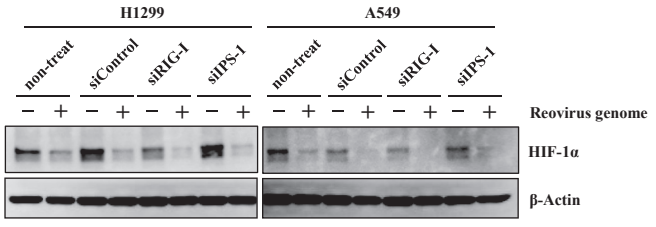

Fig. 4. HIF-1α protein levels in human cultured tumor cells pre-transfected with siRNAs targeting RIG-I and IPS-1 following reovirus genome transfection. H1299 and A549 cells were transfected with siRNAs targeting RIG-I and IPS-1. On the following day, cells were transfected with reovirus genomes at 4 ng/mL, followed by incubation for 24 h under hypoxic conditions. HIF-1α protein levels were measured by western blotting analysis.

the cells were pre-treated with cathepsins B and L inhibitors (Fig. 3), indicating that endo-lysosomal escape and recognition of reovirus genomes in the cytoplasm are important for the down-regulation of HIF-1α protein levels. In this study, treatment with the cathepsin B inhibitor CA-074 alone reduced the HIF-1α protein levels in H1299 cells under hypoxic conditions, although a previous study reported that HIF-1α protein levels were up-regulated in bovine retinal endothelial cell treated with cathepsin B inhibitor under normoxic conditions [22]. Cathepsin B would be expected to play a role in HIF-1α turnover; however, whether cathepsin B mediates up- or down-regulation of HIF-1α expression is dependent on the cell type.

Previous studies demonstrated that reovirus genomes are recognized by RNA helicase family members, including RIG-I, MDA5, the DDX1–DDX21–DHX36 complexes, and DHX9 [10,23,24]. In particular, our group reported that RIG-I knockdown resulted in an approximately 90% reduction in IFN-β mRNA levels in H1299 and A549 cells following reovirus infection, indicating that RIG-I is mainly involved in reovirus genome-induced type I IFN induction [20]. On the other hand, this study demonstrated that neither RIG-I nor IPS-1 played a crucial role in reovirus-induced HIF-1α down-regulation. Other cytosolic RNA-binding proteins would be involved in the recognition of reovirus genomes and subsequent down-regulation of HIF-1α. In addition to reovirus genomes, polyI:C efficiently down-regulated the protein levels of HIF-1α (Fig. 2A). These results indicate that relatively long dsRNA mediates the down-regulation of HIF-1α, irrespective of the nucleotide sequence. polyI:C has been used as a vaccine adjuvant in clinical and pre-clinical studies due to its strong induction of type I IFNs [25]. HIF-1α is highly involved in both innate and acquired immunity [26]. polyI:C-induced down-regulation of HIF-1α in immune cells might contribute to adjuvant effects of polyI:C.

In contrast to reovirus, several viruses, including Epstein Barr virus (EBV), Kaposi's sarcoma-associated herpes virus (KSHV), hepatitis B virus (HBV), hepatitis C virus (HCV), human papillomavirus (HPV) and human T-cell leukemia virus type 1 (HTLV-1), have been shown to induce the up-regulation of HIF-1α expression *via* several mechanisms, including activation of transcription and inhibition of degradation of HIF-1α [27–32]. HIF-1α up-regulates the expression of several virus genes [33]. Up-regulation of HIF-1α contributes to viral multiplication and transmission as well as to the promotion of tumor progression. dsRNA is a major ligand of viruses for the host defense system. The host defense system might down-regulate HIF-1α in order to suppress not only virus infection but also tumor progression by recognizing virus-derived dsRNA, although the significance of down-regulation of HIF-1α for the host defense system remains to be elucidated.

In summary, this study demonstrated that reovirus genomes and polyI:C, which are dsRNAs, induced the down-regulation of HIF-1α protein levels in a RIG-I/IPS-1-independent pathway. This study provides importance clues not only for the clarification of reovirus-mediated HIF-1α down-regulation and the optimization of reovirus virotherapy for cancers, but also for the potential development of a dsRNA-based vaccine adjuvant.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We would like to thank Sayuri Okamoto and Eri Hosoyamada (Osaka University, Osaka, Japan) for their help. This study was supported by a Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Culture, Sports, Sciences, and Technology of Japan (Grant number 26293118) and a grant from the Ministry of Health, Labour and Welfare of Japan.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.147.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.147.

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